ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 62, ART. 14 PAGES 327-348

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ROY WALDO MINER

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BY

L. JOE BERRY

NEW YORK
PUBLISHED BY THE ACADEMY
November 2, 1955

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Introduction

The survival time of mice infected intraperitoneally with a standardized suspension of one of seven different pathogenic bacteria is significantly reduced by injections of sublethal quantities of certain inhibitors or intermediates of the tricarboxylic acid cycle.[1,2,3] The most striking example of this effect occurred in two separate experiments where the infectious dose killed none of the control mice during a six-day period of observation but was uniformly fatal within 10 hours in the presence of appropriate inhibitor[3] (TABLES 5 and 6). Each of the five different compounds tested varies in its ability to alter the outcome of infections caused by different organisms. The most effective substance in the presence of one species of pathogen may have little influence in the presence of another. Injections of malonate, for instance, are accompanied by minimum survival time in animals infected with *Salmonella typhimurium*, hemolytic streptococci, *Micrococcus pyogenes* var. *aureus*, or *Proteus morganii*, while citrate injections give minimum survival time in mice infected with *Corynebacterium kutscheri*, and injections of fluoroacetate are most effective in mice infected with *Klebsiella pneumoniae*.[1,2] A given metabolic disturbance, therefore, seems to promote one infectious agent more effectively than another.

In the dosages employed, each inhibitor is known to alter significantly the citric acid concentration of selected tissues of mice.[4] This observation, in conjunction with abundant evidence from the literature on the mode of action of these inhibitors (see references in papers already cited), suggests a potential linkage between the reduction in survival time and an imbalance in metabolism of the host organism. It becomes important to know, therefore, whether an infected mouse given metabolic inhibitor or intermediate survives for a shorter period of time than control mice because of (1) more rapid *in vivo* multiplication of the pathogen, thereby permitting the lethal number of bacteria to develop within a shorter period of time; (2) a greater sensitivity to pathogens, which reduces the number required to kill, or (3) to a combination of (1) and (2).

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*This study was supported by funds provided under contract AF 18(600)-551 with the United States Air Force School of Aviation Medicine, Randolph Field, Tex.

**The author gratefully acknowledges the technical assistance of Beverly S. Morris.

It has already been shown that a more severe bacteremia appears in infected mice injected with malonate, citrate, fluoroacetate, or succinate than in control mice injected with saline alone.[5,6] The survival time of mice under these conditions is approximately inversely proportional to the logarithm of the maximum number of bacteria appearing in unit volume of blood during the first 12 hours postinfection.[6] On the basis of this evidence, it might be inferred that the bacteria reproduce more rapidly in mice with "altered metabolism," but the question remains as to how accurately quantitative evaluation of bacteremia reflects the picture in the whole animal. This is particularly problematical in light of the important qualitative studies by Webster,[7] who observed that mice with salmonellosis artificially induced via the intraperitoneal route develop two to four hours postinfection a bacteremia which persists for about 12 hours and then disappears. The blood remains bacteriologically sterile for hours or days but, near the terminal stages of the disease, it becomes reinfected and continues bacteremic until death of the animal.

In order to answer acceptably the question as to how rapidly the bacteria multiply *in vivo,* it would be necessary to follow the change with time in total population of pathogens within the body of the mouse. If the technique for accomplishing this were available, it would then be possible to determine the total number of pathogens present at death in animals succumbing to the infection under different experimental conditions. The first part of this report is concerned with this fundamental problem, and it shows, within the limits of the method, that mice infected with S. *typhimurium* die more quickly than control mice when malonate injections are administered because there is a more rapid *in vivo* multiplication of the bacteria and, consequently, a reduction in the time required to reach a lethal number.

The second part of this presentation is devoted to an analysis of why the pathogens reproduce more rapidly in mice given injections of the inhibitor, malonate, than in control mice. Two explanations are possible. Either the number of bacteria destroyed by the animal's defense mechanisms is reduced, leaving a larger growth potential, or else the *in vivo* nutritive environment on which the bacteria are dependent is enhanced, perhaps by the accumulation of metabolic intermediates, which, in turn, reduces the generation time of the microorganisms. Evidence for the former is negative, and that for the latter positive.

Methods

Determination of the number of S. typhimurium *in the whole mouse.* Mice weighing approximately 20 gm. were infected intraperitoneally at intervals of 15 minutes with about 250,000 cells of S. *typhimurium* contained in 0.5 ml. of a saline dilution of a 15-hour brain-heart infusion

(Difco) broth culture. Immediately after infection, half the animals were injected via the same route with 20 mg. disodium malonate (Eastman) dissolved in 0.5 ml. of 0.9 per cent solution of sodium chloride, and the remaining half were injected with the same volume of saline alone. In the first experiment, each mouse was sacrificed by a blow on the head one hour postinfection. The mouse was skinned, the feet and tail were removed and the carcass was weighed on a triple beam balance to the nearest quarter-gram. The carcass was then placed in a Waring blendor, to which 4 ml. of saline were added per gram of body weight, *i.e.*, a 1:5 dilution of the carcass. After blending for five minutes, the homogenate was diluted with 0.9 per cent saline. One tenth ml. of the dilution was transferred to each of four SS agar plates (*Salmonella-Shigella* agar, Difco). The plates were incubated at 37° C. for 48 hours. The number of colonies was determined per plate and the four counts were averaged. This figure, multiplied by the dilution factor, gave the number of S. *typhimurium* per mouse. The time required to carry out this procedure was less than 15 minutes. Each mouse was sacrificed, therefore, one hour postinfection.

Since any latent infection with *Salmonella* or *Shigella* would contribute to the colony count on SS agar, presumably normal mice selected at random from the colony were sacrificed from time to time during the course of these experiments, and homogenates, prepared as a 1:5 dilution, were cultured without further dilution. These were negative.

The second experiment differed from the first in the number of in jections of malonate or saline given the mice prior to sacrificing, and hence in the time postinfection at which the bacterial counts were made. Immediately after infection, each animal received the first of seven hourly injections of malonate or of saline, and the mouse was sacrificed 6.5 hours later, *i.e.*, 30 minutes after the seventh injection.

Three control experiments were included to check on this method of determining the number of S. *typhimurium*. Controls (1) and (2) were set in triplicate, and control (3) in quintuplicate.

(1). A 15-hour brain-heart infusion broth culture of the organism was grown in a volume of about 15 ml. of medium. The number of bacteria per ml. of culture was determined by means of the conventional dilution count technique. Each of four SS agar places was inoculated with 0.1 ml. of the final dilution of the culture, and the average number of colonies per plate was multiplied by the dilution factor.

(2). From the same 15-hour culture, three additional counts were made in which the first 1:100 dilution was mixed for five minutes in the the Waring blendor. All subsequent dilutions were shaken in the usual way, and each of four SS agar plates was inoculated with 0.1 ml. of the final dilution. The average of the colony counts on the four plates, times the dilution factor, gave the number of bacteria. By this means, it was possible to determine whether or not blending alters the number of viable bacteria.

(3). The final check was identical to the second except that, just before blending the 1:100 dilution of the broth culture, the carcass of a normal mouse, prepared in the manner described above for the experimental mice, was added to the blendor. Each gram of carcass was considered equivalent to one ml. of saline. Thus, if the carcass weighed 18 grams, 81 ml. of saline were added to the one ml. of broth culture, and the homogenate was prepared by blending for five minutes. Subsequent dilutions were then made according to standard procedure. The results of these control determinations are given in TABLE 1. It is apparent that acceptable agreement was obtained with the three methods.

Determination of the number of S. typhimurium *in the carcass of mice dying of the infection.* All animals used in these determinations were infected intraperitoneally with S. *typhimurium* in the manner described above. Immediately after death of the mouse, a homogenate was prepared from its carcass in a Waring blendor. A dilution count in quadruplicate on SS agar was then made with this material. Since more than a few minutes' delay between death of the mouse and the preparation of the homogenate would invalidate the count, it was necessary to keep the mice under close observation. One could expect only a small fraction of

TABLE 1

Number of *S. typhimurium* per ml. of 15-hour brain-heart infusion broth culture determined by		
Standard dilution count method	Mixing initial 1:100 dilution in Waring blendor	Mixing initial 1:100 dilution in Waring blendor in presence of mouse carcass
700,000,000 680,000,000 650,000,000	700,000,000 690,000,000 690,000,000	712,000,000 589,000,000 600,000,000 660,000,000 660,000,000
Avg. 677,000,000	693,000,000	644,000,000

Number of *S. typhimurium* per ml. of 15-hour brain-heart infusion broth culture determined by the usual dilution count method, by mixing the initial 1:100 dilution of the culture for 5 minutes in a Waring blendor followed by the usual dilution procedure, and by mixing the initial 1:100 dilution of the culture for 5 minutes in a Waring blendor in the presence of the carcass of a normal mouse followed by the usual dilution procedure. Each value shown is the average of quadruplicate counts on the same final dilution.

animals in any group to die during those periods of the day when such close supervision was possible, so determinations were carried out on animals used in connection with other experiments. Bacterial counts were made, therefore, on the carcasses of mice that had received injections of various inhibitors or intermediates at dosage levels and at intervals of time postinfection described in a previous publication.[3]

Preparation of bacteriological culture media from bodies of mice injected with malonate or with saline. Three mice were injected intraperitoneally at hourly intervals with 20 mg. of malonate dissolved in 0.5 ml. of 0.9 per cent sodium chloride solution for a total of six injections. Three animals were similarly injected with an equal volume of saline. The saline injections were started 15 minutes after the first malonate injection. The mice given malonate were killed in rapid succession by a blow on the head, starting 15 minutes after the sixth injection. The animals were skinned, eviscerated (primarily to eliminate the intestine with its mass of microorganisms), freed of loose fat, weighed to the nearest quarter-gram, and placed in an iced Waring blendor. Two ml. of ice-cold distilled water were added to the blendor per gram of animal. The carcasses were blended for five minutes, transferred to 50-ml. centrifuge tubes, and centrifuged for 10 minutes. During the period of centrifugation, the mice injected with saline were similarly prepared. The turbid supernatant from mice treated with malonate was pooled, the pH adjusted to neutrality, as measured with a Beckman line operated pH meter, and transferred to clean centrifuge tubes. The same procedure was followed with the homogenate of mice injected with saline. All tubes were then autoclaved at 15-lb. pressure for 20 minutes, recentrifuged, and the clear supernatant was transferred aseptically in 10-ml. aliquots to sterile test tubes of uniform size. These were stored overnight in the refrigerator and removed the following morning to a 37° C. incubator for one hour. One tube containing the extract from mice injected with malonate and one from mice given saline were selected for growth studies. Each was inoculated with a standardized suspension of S. *typhimurium* and, at intervals thereafter, one ml. was withdrawn for bacterial dilution counts. Quadruplicate counts were made on the final dilution, and the average number of colonies was used in calculating the total number of cells per ml. of culture. This experiment was repeated six times.

Determination of thorotrast retained by liver, spleen, and lungs of mice injected with malonate or with saline. Two tenths ml. of Thorotrast (Heyden Chemical Corp., New York, N. Y.) was injected into the tail vein of a mouse. Immediately thereafter, the first of eight hourly intraperitoneal injections of either 20 mg. malonate dissolved in 0.5 ml. saline or of saline alone was given. Ten hours after Thorotrast administration, the animal was sacrificed by a blow on the head, and the liver, spleen, and lungs were removed. Each organ was weighed promptly on a Roller-Smith balance and dried overnight in a hot air oven at 90° to 95° C. The desiccated organs were ground individually with an agate mortar and pestle, and the powder was transferred to a stainless steel pledget. Radioactive assays were carried out, using a thin-walled Geiger tube and Berkely scaler. The pledget containing the powder was placed one cm. from the window of the tube in a Tracerlab sample holder. Five successive determinations of the time required for 1,000 counts were made with each organ. The average time was then corrected for background and compared to a corrected standard prepared by desiccating 0.2 ml. of Thorotrast (taken as equivalent to 50 mg. of thorium dioxide).

General. Young adult female mice of the CF 1 strain (Carworth Farms, New City, N.Y.) from two to four months of age were used in these studies. They were housed in small galvanized iron cages with wood shavings for bedding. Their diet consisted of Purina dog chow checkers. Food and water were available to them at all times.

Results

1. *The number of* S. typhimurium *in the body of mice one hour after infection and after a single injection of either malonate or saline.* It was assumed that all colonies developing on SS agar were *S. typhimurium*. The potential error inherent in this assumption is apparent to the author. Inocula from uninfected control mice, however, failed to yield colonies on SS agar. Admittedly, some of the experimental animals may have harbored latent infections with members of the *Salmonella* or *Shigella* groups.

The number of *S. typhimurium* in 10 infected mice that had been given a single injection of 20 mg malonate and sacrificed one hour post infection is listed in the first column of TABLE 2. Individual values range from a minimum of 22,000 bacteria per mouse to a maximum approximately 10 times as great. All animals contained fewer bacteria than the number present in the infectious dose (250,000). During the initial hour of the infection, the defense system of the mouse appeared to destroy, on the average, between 2/3 and 3/4 (179,000/250,000) of the injected pathogens.

Results with 10 control mice those injected with saline, are listed in the second column of TABLE 2. The number of viable *S. typhimurium* in these animals one hour postinfection varied from a minimum of 6,500 to a maximum of 79,200, with an average of 36,000. Comparison of the bacterial counts in the two groups of mice suggests that either the defense mechanism of the control mice was approximately twice as effective as for mice receiving malonate; or, alternatively, that the bacteria in the experimental animals reproduced twice as rapidly as in the control series. Both interpretations are equally valid on the basis of these data.

Despite the fact that the average number of S. *typhimurium* in mice given malonate is nearly twice that for control mice, the difference is not statistically significant according to the rank order test of White.[8] Because of the wide variation in individual values within each group, a much larger sample would be required, assuming that the means were not appreciably altered before significance could be established.

When the average weight of the carcasses in each of the two groups of mice shown in the third and fourth columns of TABLE 2 is used to calculate the mean number of bacteria per gram of body weight, it is found that the mice injected with malonate contain 3800, and those given saline, 2000. This is shown at the bottom of TABLE 2. These values agree closely with the number of bacteria per ml. of blood, as previously reported by Berry and Mitchell,[6] in comparable groups of mice. This

TABLE 2

Estimated number of S. *typhimurium* (in thousands) in mice given		Weight of carcass (in gms.) of mice given	
Malonate	**Saline**	**Malonate**	**Saline**
22.2	6.5	18.5	16.25
22.2	7.0	18.5	17.5
31.2	7.4	19.5	18.5
31.2	22.2	19.5	18.5
35.5	32.0	17.75	16.0
41.0	43.2	20.5	18.0
51.2	46.2	16.0	19.25
120.0	49.2	20.0	20.5
128.0	65.7	17.75	18.25
223.0	79.2	18.0	18.0
Avg. 71±63	36±24	18.6	18.1

$$\frac{71/18.6}{36/18.1}=\frac{3800}{2000}=1.9$$

ratio of number of bacteria present per gm. in mice given malonate to number present per gm. in mice given saline

Estimated number of S. *typhimurium* in the carcass of individual mice following intraperitoneal injection of either 20 mg. malonate in 0.5 ml. saline or 0.5 ml. saline alone. Injections were given immediately after intraperitoneal infection with about 250,000 cells of S. *typhimurium*. All mice were sacrificed one hour postinfection.

agreement indicates that intraperitoneal infection within the first hour gives rise to a relatively uniform distribution of bacteria throughout the body of the mouse. The blood at this time appears to be in an "equilibrium" state, containing neither more nor less bacteria than other tissues of the body.

2. *The number of* S. typhimurium *in the body of mice 6.5 hours after infection and after seven injections of either malonate or saline.* The first column of TABLE 3 lists the number of *S. typhimurium* in the body of each of 15 mice 6.5 hours after intraperitoneal infection with approximately 250,000 cells and after seven hourly injections of 20 mg. malonate. The smallest total count was 17.6 million, the largest was 54.7 million, and the average was 32.1 million. Bacterial counts for the same number of mice similarly infected but given seven injections of saline are presented in the second column of TABLE 3. In these animals, the minimum number was 550,000, the maximum was 4.62 million, and the mean was

TABLE 3

Estimated number of *S. typhimurium* (in millions) in mice given		Weight of carcass (in gm.) of mice given	
Malonate	Saline	Malonate	Saline
17.6	0.55	12.75	15.25
17.7	0.78	15.25	15.0
18.5	0.81	16.0	15.5
23.0	0.81	18.0	21.25
23.5	0.93	15.75	20.0
27.0	1.13	15.75	15.75
29.6	1.16	15.0	15.25
30.4	1.19	16.0	18.75
30.5	1.55	16.0	15.5
34.0	2.04	18.75	16.5
36.0	2.29	15.25	12.75
41.2	2.47	13.75	15.25
45.2	2.93	15.25	15.25
52.2	3.42	14.5	16.0
54.7	4.62	13.75	13.5
Avg. 32.1±11.5	1.65±1.11	15.5	16.0
$\frac{32.1/15.5}{1.65/16.0} = \frac{2.08}{.103} = 20$		In blood,* $\frac{.3441}{.0181} = 19$	

Estimated number of *S. typhimurium* in the carcass of individual mice following seven intraperitoneal injections of either 20 mg. malonate in 0.5 ml. saline or 0.5 ml. saline alone. Injections were given at hourly intervals beginning immediately after intraperitoneal infection with about 250,000 cells of *S. typhimurium*. All mice were sacrificed 6.5 hours postinfection.

*Berry and Mitchell[6]

TABLE 4

Survival time—hours postinfection	Type of injections given postinfection	Number of S. *typhimurium* in body of mouse at time of death
4.5	malonate*	93,000,000
5.5	malonate*	101,000,000
13.	succinate*	100,000,000
29.	fluoroacetate**	360,000,000
29.	malonate*	297,000,000
29.5	fluoroacetate**	180,000,000
30.	fluoroacetate**	303,000,000
30.	malonate*	205,000,000
31.	malonate*	83,000,000
31.	succinate*	103,000,000
32.	fluoroacetate**	243,000,000
49.	fluoroacetate**	73,000,000
54.	malonate*	176,000,000
54.5	malonate*	180,000,000
71.	saline***	63,000,000
71.	citrate****	63,000,000
199.	none	172,000,000
219.	none	113,000,000
		avg. 162,000,000±87,600,000

Estimated number of S. *typhimurium* in the body of artificially infected mice at time of death. Each animal was infected intraperitoneally with about 250,000 cells.

*20 mg. hourly for 8 injections (if mice survive).
**0.125 mg. every 6 hours for 2 injections.
***0.5 ml. hourly for 8 injections.
****10 mg. hourly for 8 injections.

1.65 million. The probability that the difference between the means of the two groups occurred by chance is less than .001 by the rank order test.[8]

The ratio of the number of bacteria per gram of body weight in mice injected with malonate to the number per gram in mice given saline, based on average weights of the two groups of animals as shown in the third and fourth columns of TABLE 3, is 20 (see bottom of TABLE 3). Under comparable experimental conditions, the ratio of the number of bacteria per ml. of blood in mice injected with malonate to the number in mice injected with saline (data from Berry and Mitchell[6]), is 19 (values given at bottom of TABLE 3). It can be concluded, therefore, that quantitative evaluation of the bacteremia in mice of the two groups yields, at this time postinfection, a reliable estimate of the *relative* severity of the infection, but it fails to give an accurate estimate of *absolute* numbers since blood in both groups of mice contains per ml. only about one sixth as many bacteria as the entire mouse contains per gram (.3441/2.08 and .0181 .103). Thus, in the interval between one and six and one half hours after infection, the blood changes from a condition in which it is in "equilibrium" with the bacterial population of the animal as a whole (see section above) to one in which it contains fewer bacteria. This latter situation could exist only in the presence of a mechanism which either actively prevents entrance of bacteria into the circulatory system or eliminates them faster from it. Whatever the mechanism may be, it is *equally* effective in eliminating *proportionate* numbers of bacteria in the two groups of mice, even though animals injected with malonate are loading the system with 20 times as many bacteria. This is apparent from the calculations 2.08/.108 = 20 (cited above), yet the blood of both groups of mice contain one sixth as many bacteria per ml. as the mice contain per gram. If the phagocytic cells of the reticuloendothelial system are responsible for this process, their functional integrity appears to be uninfluenced by injections of malonate. Evaluation of Thorotrast uptake by liver, spleen, and lung, described in section 4 below, leads to the same conclusion.

3. *The number of* S. typhimurium *in the body of mice immediately after death.* The number of S. *typhimurium* in the body of infected mice was determined immediately after death of each animal and the results are listed in increasing order of survival time in the third column of TABLE 4. The time of death, given in the first column, ranges from 4.5 hours to 219 hours postinfection. The type of injection each animal received is indicated in the second column. The minimum number of bacteria at death is 63 million, the maximum number is 360 million, and the average is 162 million. This is considered a small variation in individual values, particularly since the mice employed must be assumed genetically heterogeneous (the colony is maintained by out-breeding) and since a

TABLE 5

Organ	Thorotrast content of organs (in mg.) of mice given					
	Malonate			Saline		
	Number of mice	Average ± standard deviation	Range	Number of mice	Average ± standard deviation	Range
Liver	16	12.0 ± 3.9	7.5 - 23.0	18	13.4 ± 3.5	8.7 - 20.4
Spleen	16	11.7 ± 4.4	5.5 - 18.5	18	13.7 ± 4.0	8.0 - 20.2
Lungs	17	1.0 ± 0.4	0.4 - 1.6	18	1.2 ± 0.4	0.6 - 2.2

Retention of Thorotrast in liver, spleen, and lungs of mice given eight hourly injections of either 20 mg. of malonate dissolved in 0.5 ml. of saline or of 0.5 ml. of saline alone. Each animal was injected intravenously with 0.2 ml. of Thorotrast at time "O" followed immediately by the first injection of malonate or of saline. All mice were sacrificed at 10 hours.

TABLE 6

Date of experiment	Age of culture (in hours)	Number of bacteria (in millions) in medium made from mice given		Percentage difference in growth
		Malonate	Saline	
12/2	5.5	31.3	15.6	200
12/9	6.0	45.0	14.0	322
12/15	5.5	20.8	17.0	122
1/5	5.5	37.8	22.0	172
1/12	5.5	46.0	35.0	153
2/10	6.5	54.0	22.0	245
Average	5.8	39.2 ± 10.8	20.9 ± 7.0	202

The number of S. *typhimurium* appearing after 5.5 to 6.5 hours of growth at 37° C. in culture media prepared from the eviscerated carcasses of mice 15 minutes after the seventh hourly injection of either 20 mg. ·malonate dissolved in 0.5 ml. of saline or 0.5 ml. of saline alone.

number of similar survival curves following intraperitoneal infection with S. *typhimurium* have been obtained with mice of this strain in which the first deaths occur on the third day postinfection, the last deaths occurring after two weeks or longer (*cf.* Figure 2, Berry and Mitchell[9]). *The primary variable entering into the survival time of infected mice, with and without injections of metabolic inhibitors and intermediates, seems to be, therefore, on the basis of the data of* TABLE *4, the time required for a relatively constant lethal population of pathogens to be reached rather than a change in sensitivity of the animal to a given number of microorganisms.*

An interesting question is posed by the results with the mouse represented in the first row of TABLE 4. Death occurred at 4.5 hours, and the bacterial count was 93 million. What kind of bacterial generation time would be required within the mouse in order for this to occur? If it is assumed that the animal, at the end of the first hour postinfection, had the maximum number of S. *typhimurium* shown for the mice listed in TABLE 2 (223,000), then, in order for 93 million to appear within the next 3.5 hours (210 minutes), there would have to be 8.7 generations. This gives a generation time of 24 minutes, a time very close to that found for this organism grown in brain-heart infusion broth (see the first row of TABLE 7). The nutritive environment within this particular mouse must have been unusually favorable for growth of S. *typhimurium*.

On the basis of the data presented in this section, it might be argued that several of the mice injected with malonate and sacrificed for the bacterial counts listed in TABLE 3 were within an hour or two of death. Counts of 40 to 50 million, after one to four additional generations would yield a lethal population of bacteria.

4. *Retention of Thorotrast in organs of mice injected with either malonate or saline.* In section 2, above, evidence was given that suggested that the capacity of the phagocytic cells of the reticuloendothelial system for removal of bacteria was as effective in mice administered a series of injections of malonate as in mice injected with saline. In order to obtain more specific information on this point, the experiments now to be described were undertaken. Thorotrast, introduced parenterally, is known to be ingested by the fixed and wandering macrophages of various organs, but particularly by those in spleen and liver. According to Easton,[10] there is a gradual excretion of the insoluble thorium dioxide, probably from the liver via the bile into the intestinal tract and from the lungs into the saliva. In most of the earlier reports in the literature, the time elapsing from the injection of Thorotrast to sacrifice of the animals has been at least 24 hours and, in many cases, longer. Since the effect of malonate injections on the course of bacterial infections is already extensive after a period of 6.5 hours, it was deemed necessary to evaluate the action of this inhibitor on the reticuloendo-

TABLE 7

Bacteria grown in	Number of bacteria per gm. or per ml. of "medium"		Elapsed time in minutes	Number of generations	Generation time in minutes
	Initial count	Final count			
Brain-heart infusion broth	6,600	196,000,000	330	14.9	22.1
Mice given saline	2,000*	103,000**	330	5.7	58.0
Mice given malonate	3,800*	2,080,000**	330	9.1	36.3
Blood of mice given saline	2,100***	18,100***	300	3.1	96.8
Blood of mice given malonate	1,300***	344,100***	300	8.0	37.5
Medium made from mice given malonate	18,000	39,200,000****	258	11.1	24.3
Medium made from mice given saline	15,000	20,900,000****	258	10.4	24.8

Generation times of *S. typhimurium* under different *in vitro* and *in vivo* growth conditions.

*Data from TABLE 1.
**Data from TABLE 2.
***Data from Berry and Mitchell.[6]
****Data from TABLE 5.

thelial system for the briefest possible time. Accordingly, the eight injections of malonate or saline, the number usually given in the infection experiments,[1,3] were completed seven hours after the intravenous administration of Thorotrast and, three hours later, the animals were sacrificed. The average quantity of thorium dioxide found in each of the three organs of 16 to 18 mice given one of the two types of injections is shown in TABLE 5. In every case, the average is slightly higher for organs of control mice, but differences of this magnitude would occur by chance alone in about 50 per cent of the cases. Statistically, therefore, no significant effect can be deduced from these experiments, and it is concluded that malonate does not interfere with the phagocytic action of cells involved in the uptake of Thorotrast.

5. *Growth of* S. typhimurium *in culture media prepared from mice injected with malonate or mice injected with saline.* The results of six separate experiments, in which the growth of S. *typhimurium* in a medium prepared from the eviscerated carcasses of mice injected with malonate is compared with growth in a medium similarly prepared from mice given saline, are presented in TABLE 6. For each experiment, the values represent the maximum difference in numbers of bacteria observed after an incubation period of 5.5 to 6.5 hours, the maximum occurring at 5.8 hours on the average. In every case, more bacteria appear in the medium prepared from malonate-injected mice, the mean being almost twice that for a medium made from mice given saline. The difference is statistically significant, with a probability between .01 and .05 as calculated by the rank order test.[8] This is in agreement with inferences drawn from the experiments described in previous sections of the report but, despite the confidence implied by the statistical test, the difference is small for bacterial counts of the magnitude involved. Adding to the uncertainty is the transitory nature of the difference. Usually, after 8 hours of incubation, and always after 24 hours, the same average number of bacteria was found in the two types of media.

Of equal importance in this connection, however, are the following considerations. If it is assumed that malonate, by inhibiting certain steps in host metabolism, leads to an accumulation of one or more intermediates that become available for bacterial growth, then each injection of the inhibitor would permit production of a limited supply of nutritive material. It would be available for only part of the postinjection period, since malonate is known to be both oxidized and excreted by mice.[11-14] It is also known that if malonate injections are limited to four (rather than eight), or if they are given before infection, the survival time of mice is not affected.[1] If improved bacterial nutrition due to malonate is to account for the enhancement of the infection, it must be the result of small increments of growth repeated with each injection of the inhibitor.

Should such a concept have validity, then the twofold growth differential observed, on an average, in TABLE 6, might be as much as one could anticipate. Once the limited supply of accumulated intermediate(s) is depleted, utilization of other "extractives" in the medium might mask the initial transitory difference in growth rates.

6. *Calculation of the generation time of* S. typhimurium *under* in vivo *and* in vitro *conditions*. The data of TABLES 2, 3, and 6, along with those from a previous publication,[6] have been utilized for the calculation of generation times of *S. typhimurium* under various *in vivo* and *in vitro* conditions. The necessary information and the results are given in TABLE 7.

The first row, referred to in section 3 above, shows a generation time of 22.5 minutes for growth in brain-heart infusion broth. Rows 2 and 3 contain the figures for the average number of bacteria per gram of mouse given saline or malonate, respectively, after one hour and after 6.5 hours (TABLES 2 and 3) and yield generation times of 58 and 36.3 minutes, the latter for mice given the inhibitor. The smaller value (36.3 minutes) indicates that the nutritive environment for bacteria within these mice is less favorable than that within the mouse described in section 3 (generation time, 24 minutes), but it is more favorable than that within control (saline-injected) mice (58 minutes). These calculations involve the inherent assumption that all organisms enumerated at time "zero" give rise to the final number. Actually, one might reasonably expect the defenses of the animal to destroy some of the cells. Should this be true, the real generation times would be smaller than those given in rows 2 and 3 of TABLE 7.

Rows 4 and 5 contain the data necessary for determining generation times of S. *typhimurium* within the blood stream of mice given saline (row 4) and of mice given malonate (row 5). The latter value agrees closely with that obtained for the whole mouse injected with malonate (*cf.* rows 3 and 5), while control mice have generation times that differ by more than 50 per cent when the same comparison is made (row 2 versus row 4). Since the bacteremia of control mice tends to disappear by about 12 hours postinfection (Berry and Mitchell[6]), factors seem to be operating that make this particular calculation of generation time of questionable value. It is impossible to decide, on the basis of information available, whether the generation time of 96.8 minutes is due primarily to poor bacterial nutrition or to excessive removal of bacteria from the blood stream. The latter seems more likely.

Bacteria grown in the media prepared from the carcasses of mice given injections of either malonate or saline have relatively short generation times, as shown in rows 6 and 7 of TABLE 7. The times differ by only 0.5 minute, despite the larger number of bacteria developing in the medium from malonate-injected mice, and compare favorably with that found for cells grown in brain-heart infusion broth (row 1).

Discussion

The results described in this report offer new insight into the mechanisms underlying the reduction in survival time of intected mice whose metabolism is partially inhibited by a series of injections of sublethal quantities of sodium malonate compared to the survival time of infected control mice.[1,3] It is now established that cells of S. *typhimurium* multiply more rapidly in the bodies of poisoned mice, and this, in turn, shortens the time required for the total population of these organisms to reach the lethal range. The narrowness of the range over which this pathogen is lethal for mice both with and without artificially induced metabolic disturbance was one of the unforeseen results of the investigation. It would be interesting to know whether or not these relationships have general applicability for a variety of pathogenic bacteria. The use of appropriate selective media should make possible an answer.

The accelerated growth rate of the bacteria within the experimental animals appears to be correlated, at least in part, with the internal nutritive environment on which the organisms are dependent. Evidence for this suggestion is positive and statistically significant, even though less convincing than one might wish. If a heat-stable intermediate, accumulating hypothetically as a result of malonate injections and appearing in the "mouse extract" medium could be identified, then repeated additions of this substance to a medium prepared from control animals should provide the information necessary to refute or substantiate the tentative interpretation. On the other hand, additional experiments are also indicated before the possibility of a diminished capacity for the destruction of bacteria is abandoned as an alternative explanation for the greater growth rate of bacteria in mice with altered metabolism. Retention of Thorotrast by cells of the reticuloendothelial system has been used[15-18] as a means of evaluating its phagocytic activity and, in this laboratory, the same technique was utilized to show that the spleens of anemic mice were more active than those of normal mice,[9] in confirmation of earlier *in vitro* phagocytic measurements with blood and peritoneal exudates from animals of each type.[19,20] The question nevertheless remains as to the true significance of these tests. The cellular defense might be reduced not because of an impaired capacity for ingesting particles, but rather for destroying the organisms ingested. Thorotrast would fail to reveal a deficiency of this type. It is also possible that the bactericidal power of normal blood serum recently discussed by Pillemer *et al.*[21] is diminished by injections of malonate. Until more knowledge becomes available, both the direct evidence and the inferences presented in this report must be considered tentative. Whatever the final explanation, it must account, however, for the way in which injections of an inhibitor (malonate) can enhance an infection with *S. typhimurium* and other organisms more effectively than some other inhibitor or an intermediate (such as citrate), while the reverse situation prevails in infections with other organisms (for example, *Corynebacterium kutscheri).*[1,3] It is not inconsistent with the hypothesis already advanced to attribute these observations to the comparative complexity in nutritive demands of different pathogens.

Three types of evidence are contrary to the concept that injections of malonate reduce survival time through some type of bacterial selectivity or virulence enhancement:

(1). Cultures of *S. typhimurium,* grown in brain-heart infusion broth containing the same concentration of malonate that would result if it were uniformly distributed throughout the mouse immediately after an injection had the same total number of cells per ml. as the control culture without malonate. Mice infected with the standard dilution of this culture had the same mortality as mice infected with an equal number of bacteria from the control culture.

(2). Colonies of *S. typhimurium* isolated from a mouse that had died of an infection during a series of malonate injections were transferred to tubes of brain-heart infusion broth. Mice infected with the standard number of cells from these cultures had the same survival times as mice infected with the stock culture.

(3). The absence of detectable differences in numbers of bacteria present in the carcasses of mice at death, regardless of the substance injected during the initial postinfection period (see data of TABLE 4) makes it seem unlikely that a significant change in virulence occurred. These results, taken singly or in combination, are not sufficient basis for eliminating entirely the possibility of some type of bacterial selectivity during the injections of inhibitors or intermediates, but more subtle tests will be required to detect it.

The only previous studies concerned with the influence of changes in host metabolism induced by chemical means on response to infections, insofar as the author is aware, have been with viral agents. Synthesis of some types of virus is inhibited by injections of fluoroacetate[22,23] while other types are only slightly affected.[24] Multiplication of virus has never been reported as stimulated by any antimetabolite in the way *S. typhimurium* is by malonate. Perhaps this can be attributed to the direct dependence of viruses upon the active metabolism of host cells while bacteria are metabolically independent.

The technique employed in this work for determining the number of S. *typhimurium* within the mouse during different stages of an infection might profitably be applied to a variety of problems associated with infectious diseases. For example, although much has been written about the effect of therapeutic agents on the multiplication of pathogenic bacteria in particular parts of the host—blood, peritoneal cavity, *etc.*—no information is available on their effect on the entire bacterial population of the host. The technique might also be applied to advantage in studies of the mechanisms by which stresses, such as the hypoxia of altitude, increase susceptibility to infection.[9]

An interesting calculation is made possible by knowing the mean number of S. *typhimurium* required to kill a mouse (TABLE 4). The size of individual cells of this species is 0.5 × 1.0 to 1.5 μ.[25] Determining cell volume as if it were a perfect cylinder and multiplying by the specific gravity, 1.0 to 1.1,[26] the weight of each cell amounts to 2 to 3×10^{-10} mg. The weight of 162 million cells would then be about 30 to 40 μg. A mouse of approximately 20 gm. is killed, therefore, by this comparatively small mass of vegetative cells, the dry weight of which would amount to no more than one part in a million. The toxicity of the endotoxin is several times greater than that of such potent enzyme inhibitors as fluoroacetate and arsenite. This implies that endotoxins are even more effective enzyme poisons.

Summary

1. A technique is described by means of which the total number of *Salmonella typhimurium* is quantitatively evaluated in the body of artificially infected mice at different stages of the disease. The carcass of the animal is blended in a Waring blendor for five minutes in the presence of four ml. of saline per gram of body weight. A bacterial dilution count is then made with this material, using SS agar as the medium. Control counts on a bacterial culture showed that neither the blending nor the presence of a mouse carcass altered, appreciably, the number of bacteria obtained by the conventional dilution method.

2. Mice infected intraperitoneally with a standardized suspension of S. *typhimurium* containing 250,000 cells, followed immediately by an injection of either 20 mg. malonate dissolved in 0.5 ml. of saline or saline alone, contain respectively, after one hour, an average of 71,000 pathogens (range 22,000 to 223,000), and an average of 36,000 (range 6,500 to 79,200). The difference is not statistically significant. When each type of injection is repeated at hourly intervals in similarly infected mice, the animals contain respectively, after 6.5 hours, an average of 32.1 million (range 17.6 million to 54.7 million) and an average of 1.65 million (range

0.55 million to 4.62 million). The difference is highly significant, statistically, and it indicates that the bacteria multiply more rapidly in infected mice given a series of malonate injections than in mice injected with saline.

3. The number of S. *typhimurium* was determined in the carcass of 18 mice immediately after death from the infection. All animals were inoculated initially with the same infectious dose, but survival time ranged from 4.5 hours to 219 hours, depending upon the type of injections of either metabolic inhibitor or intermediate administered. The mean number of bacteria was 162 million (range 63 million to 360 million). Duration of survival seems to depend, therefore, on the time required for the pathogens to increase until they reach a comparatively constant lethal number.

4. The greater rate at which S. *typhimurium* multiplies in mice given a series of injections of malonate, compared to that in control mice, could not be attributed to an experimentally detectable difference in the cellular defense of the mice. Equal amounts of intravenously injected Thorotrast was retained in spleen, liver, and lungs of mice injected with either malonate or saline. There was a measurable difference in growth rate of bacteria in media prepared from the carcasses of mice injected with either malonate or saline. Twice as many S. *typhimurium*, after 5.5 to 6.5 hours of incubation, were present in the medium made from mice given malonate, suggesting that a metabolic intermediate accumulates and thereby enhances the nutritive properties of the medium.

5. Generation times of S. *typhimurium* were calculated for different conditions of growth. The fastest rate, 22.1 minutes, was observed in brain-heart infusion broth. In media prepared from mouse carcasses, generation times of 24 to 25 minutes were obtained, whether or not the mice had been treated with malonate. Generation times *in vivo*, that is in living mice, were 36.3 minutes in mice given malonate, and 58 for those given saline.

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